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14. ABSTRACT

It is estimated that there will be 229,060 new cases of invasive breast cancer in the U.S. this year. One of the most aggressive phenotypes, triple negative breast cancer (TNBC) accounts for almost 20% of all breast cancer lesions. Currently, the most effective treatments involve the use of chemotherapeutic drugs such as anthracylines (e.g., doxorubicin) or taxanes (e.g., Taxol); however, despite the sensitivity of TNBCs to these drugs, the overall prognosis is still quite grim as a result of rapid development of resistance and disease recurrence (both locally and distally). For the women diagnosed with TNBC, the most critical aspect of treatment arises from the need to eliminate recurrence and metastasis, which are 15% more likely to occur in TNBC than other forms of breast cancer. Local delivery of chemotherapeutic anthracycline drugs, such as doxorubicin, provides a method by which the concentration of drug at the tumor can be elevated, while simultaneously maintaining low plasma levels of the agent thereby reducing toxicity limitations for the therapy. Additionally, the development of in situ forming implant systems (which are liquid solutions outside of the body, but solidify on contact with an aqueous environment) provides a means by which the implants can be administered under image guidance through a simple injection. While these systems provide an exciting alternative to systemically delivered therapies, they currently cannot address the underlying issue of metastasis. The overall goal of the proposed research is to develop a novel release system designed specifically to treat metastatic breast cancers, and to integrate this release technology into clinical trials for women suffering from triple-negative breast cancer (TNBC). The current research I have done has focused on the development of a local delivery system designed to release chemoattactants in order to direct cell migration toward elevated drug concentrations in order to improve the clinical outcome of patients diagnosed with TNBC by actively targeting cells that have a higher propensity for chemotaxis. Successful completion of the proposed work may alter the focus of controlled release systems away from treatment of the primary mass, and towards the treatment of the more clinically relevant target of metastatic cells.

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Section I – Progress

Overview

Since the award start date, I have developed a mathematical model in order to more efficiently evaluate the effect of the implant release profile on the spatial distribution of drug within the tissue phantom system that I have developed. This model can be easily modified to account for loss due to cellular consumption or uptake by the microvasculature. The resultant spatial distribution profile of drug released by in situ forming implants was then compared to the predicted spatial distribution profiles of the model system. From this model analysis, the desired release profile could be approximated, to aid in adjusting implant formulation parameters. The baseline release profile of doxorubicin was evaluated over the course of 21 days using two different polymer formulations: a slow degrading, highly crystalline 85:15 (lactic acid:glycolic acid) poly(lactic-co-glycolic acid) (PLGA) and a more rapidly degrading, amorphous 50:50 (lactic acid:glycolic acid) PLGA. The effect of excipients (water, BSA, DiI, and BSA-DiI) was then evaluated to determine the best method for modulating release to obtain a profile of burst release followed by near-zero order release. Additionally, the phase inversion, swelling, erosion, and microstructure were characterized. Upon completion of these studies, 2D chemotaxis studies were performed to identify the ideal chemoattactant for use in these implants. After comparing the chemotaxis results of SDF-1 and EGF (TGF- β was eliminated as a candidate chemoattractant due to the potential for converting healthy normal cells into mesenchymal-like cells through the endothelial-to-mesenchymal transition). After evaluating chemotaxis, the expression levels of CXCR4 in various cell lines was measured, and the effect TGF-β on receptor levels was also evaluated. I began development of a system to control the spatial distribution of the agent for 3D chemotaxis studies. In anticipation of evaluating the effect of SDF-1 gradients on cell migration in 3D, I made GFP positive cells and embedded them into 3D culture. IACUC protocol were also written and approved, these protocols were then submitted to ACURO, but revisions are currently pending due to change in institution. This protocol will be modified relative to the ACURO suggestions and resubmitted at the University of Michigan. Due to denaturation and poor release of growth factor from the polymer implants, a scaffolding system developed in the Lahann lab is being used to facilitate the release of growth factor. In order to evaluate the ability to elicit chemotaxis, SDF-1 was conjugated to the scaffold through the use of chemical vapor deposition, and subsequently coated with extracellular matrix proteins. Cells were then allowed to invade the constructs. To facilitate release from the scaffolds, a method for conjugating heparin to the scaffolds was developed. Conjugation was first tested using microparticles, and the effect of SDF-1 loading conditions were evaluated. Currently we are determining the release rate of SDF-1α and SDF-1γ (and ratios of the two), from the heparin conjugated scaffolds which will then be embedded in collagen gels to demonstrate the chemotaxic potential of the scaffolds.

Mathematical model of spatial distribution –

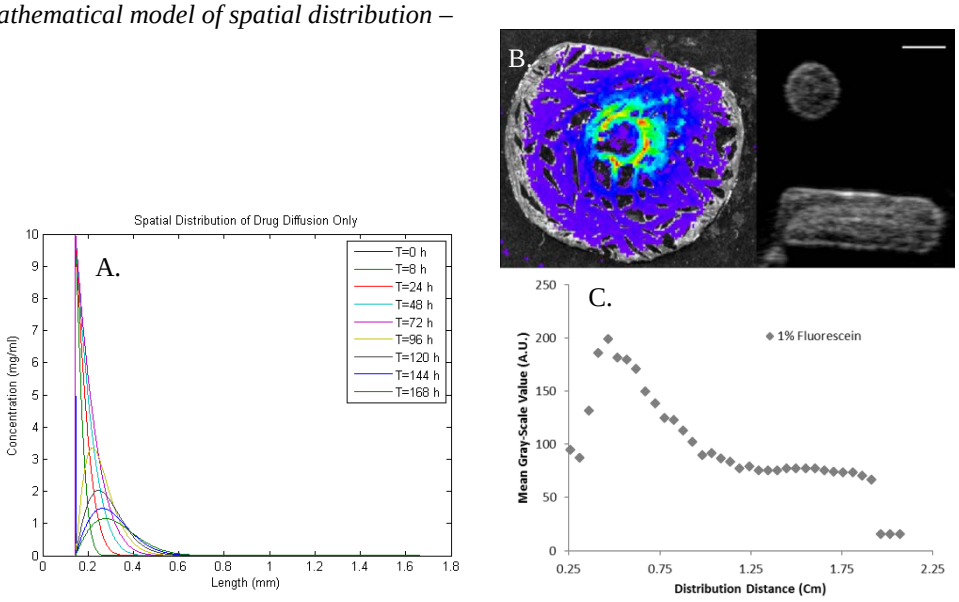

Figure 1: (**A**) Mathematical model output which can be fit to approximate spatial distribution profile of fluorescein release into gelatin. (**B**) Fluorescence image of fluorescein released from an in situ forming implant rod distributed in gelatin phantom, scale bar represents 2.5mm. (**C**) Spatial distribution profile obtained from fluorescence imaging showing the spatial distribution of fluorescein from the implants into a gelatin phantom.

The system measures the drug release within a gelatin phantom in which the implant is a polymer rod with a length greater than the diameter of the rod, allowing for the simplifying assumption that the diffusion occurs predominantly in the radial direction. Since there are no cells in the gelatin phantom, the transport equation simplifies to a model for simple diffusion. Finally, the radius of the drug eluting gelatin rod is assumed to be in quasi-steady state and not changing dimensions at a significantly fast rate. The resultant equation can be simplified to the following form, with $\frac{\partial c}{\partial t} = \frac{v_g}{r}\frac{\partial c}{\partial r} + D_g\frac{\partial^2 c}{\partial r^2}$ used to describe the change in concentration with time in the fluorescein loaded gelatin region, and $\frac{\partial c}{\partial t} = \frac{v_p}{r}\frac{\partial c}{\partial r} + D_p\frac{\partial^2 c}{\partial r^2}$ describing changes in concentration in the gelatin phantom region. The D_p is the diffusivity of fluorescein in the cross-linked gelatin phantom, and the D_g is the diffusivity of fluorescein in the gelatin rod region. The initial conditions are that the concentration is 0 in the gelatin phantom at time zero, and the concentration is 10mg/ml within the gelatin rod region at time zero. The boundary conditions are that at the center of the gelatin rod we apply the symmetry condition $\frac{\partial c}{\partial r}|_{r=0} = 0$, and at the length of the phantom $\frac{\partial c}{\partial r}|_{r=\infty} = 0$. At the interface between the rod and the phantom, we apply the flux condition such that $\frac{\partial c}{\partial r}|_{r=r_p} = \frac{v_g}{v_p}\frac{\partial c}{\partial r}|_{r=r_g}$ and $C_g=\lambda C_p$, where λ is the partition coefficient between the two phases. By altering the concentration at the gelatin border to evaluate the effect of the release profile on spatial distribution it can be observed that with burst release followed by significantly slower diffusion facilitated release, the bulk of drug shifts away from the implant surface and into the phantom (**Figure 1A**). This shift can be eliminated if zero order release follows the initial burst. This prediction is observed in the physical system (**Figure 1B** and **1C**), indicating that ideal release to develop gradients within the tissue would be a burst followed by linear release.

Release of doxorubicin from in situ forming implants –

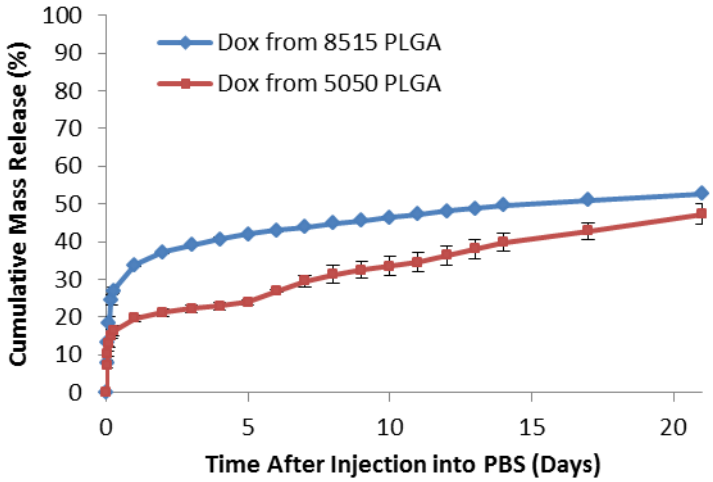

Figure 2: Release of doxorubicin from implants made using 85:15 PLGA compared with release from 50:50 PLGA implants over the course of 21 days.

A significantly higher burst was observed from 85:15 implants, with significantly slower diffusion-facilitated release over the remainder of the time course. 50:50 implants had a significantly lower burst release, but cumulative release approached what was observed in 85:15 implants after 21 days. The 50:50 polymer was then chosen to use due to the better daily release rate after burst. It was anticipated that an increased rate of drug release might be required for effective cell recruitment, so the effects of various excipients were then evaluated to determine a technique to achieve improved release from the implants. Fluorescein was used as a mock drug because it has a similar release profile to doxorubicin over the course of 14 days, but is both lower in cost and safer to use.

Effect of excipients on implant behavior and release –

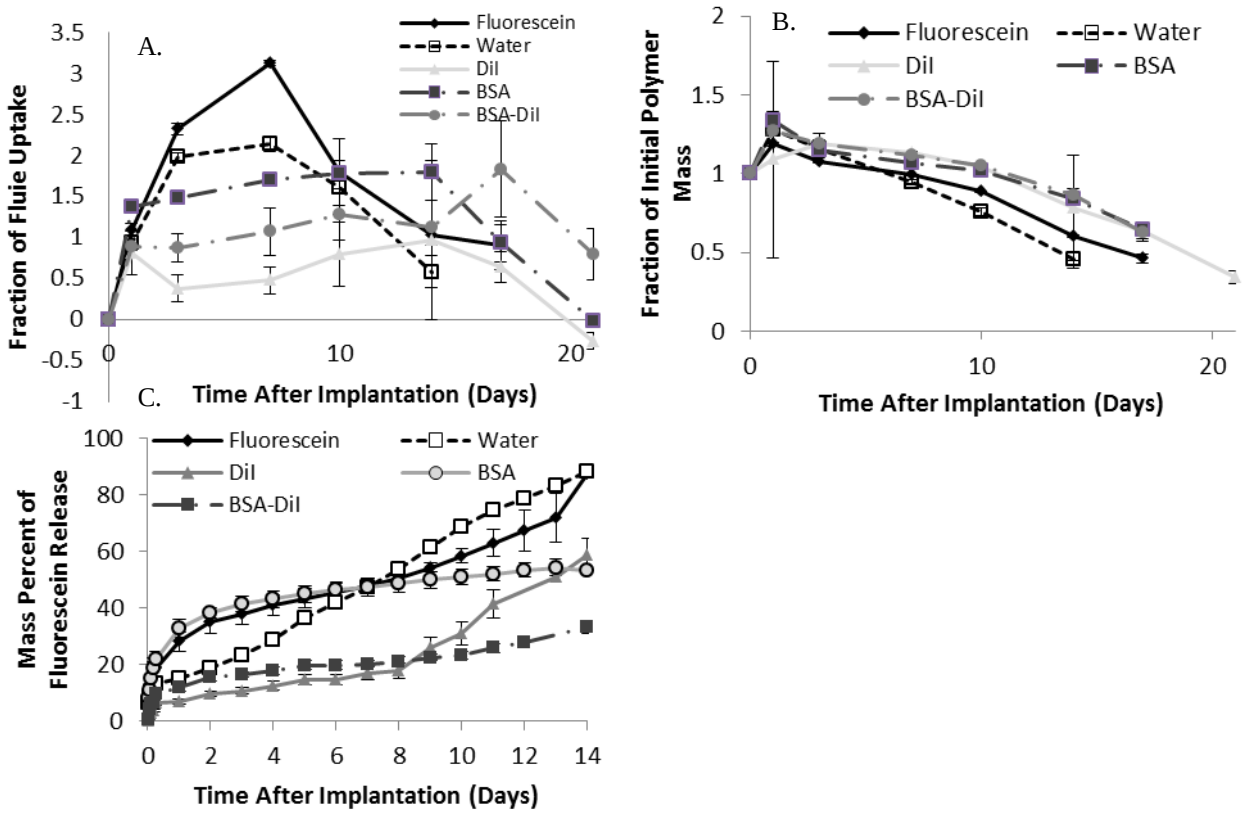

Figure 3: (**A**) Fluid uptake profile of implants loaded with various excipients. (**B**) Mass loss over time of the implants. (**C**) Effect of excipients on the releae profile of the implants. Fluorescein is the control implant with no other excipients added. Water was added to the solvent (N-Methyl-2-pyrrolidone, NMP) at a concentration of 10% water in NMP, then this solution was used to make the implants. 4% BSA was added to the polymer solution for the BSA implants, 4% 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI) was added to the polymer solution for the DiI implants, and 2% BSA and 2% DiI was added for the BSA-DiI implants.

Water was evaluated as a potential excipient because it was anticipated that the resultant change in nonsolvent/solvent gradient would lead to a more homogenous spongy microstructure with a thinner outer shell region leading to a shell with a lower diffusivity and more zero order release behavior. BSA was added to determine what effect coloading protein with drug might have on release. It was anticipated that the protein would electrostatically interact with the polymer, reducing the erosion rate and subsequently decreasing fluorescein release. DiI was added to evaluate the effect of increased hydrophobicity on release. It was anticipated that the hydrphobic agent would decrease water uptake and reduce burst. The combination of DiI and BSA was evaluated to see if burst could be manipulated in the presence of protein. Water, BSA, and DiI all appeared to lower the uptake of PBS into the implants (**Figure 3A**). It was anticipated that BSA and BSA-DiI would decrease the rate of polymer erosion, while water and DiI would increase the rate (due to previous studies performed evaluating DiI alone). We observed that water increased the rate of erosion relative to the fluorescein controls, but DiI also decreased the rate of erosion, which we hypothesize was a result of the decreased water uptake leading to reduced hydrolysis (**Figure 3B**). When comparing the release rate of the control fluorescein alone implants with those containing excipients, it was observed that the addition of water significantly reduced the burst release, while facilitating near linear release after burst. Addition of BSA did not alter the burst phase of release, but delayed the onset of degradation facilitated release by several days, which we hypothesisze to be a result of the polymer/BSA electrostatic interactions. DiI could be used to reduce the initial burst, but did not appear to alter the onset of degradation facilitated release. The combination of BSA and DiI both reduced burst and delayed the onset of degradation facilitated release (**Figure 3C**). To understand the potential mechanism of release and erosion, SEM images were take to evaluate the effect on microstructure. We observed that implants containing BSA and BSA-DiI both had an outer shell thickness comparible to the fluorescein implants, but both had a much denser internal structure than implants made using fluorescein alone. Those containing water had a lower density internal structure as well as a thinner outer shell, which we hypothesize leads to the observed release profile. DiI implants had large discontinuous macropores, with regions of dense polymer between the macropores, which we hypothesize reduces burst due to poor diffusion pathways (**Figure 4**). Due to the improvement in

release profile, water will be used to modify the release profile of the implants to facilitate elevated release of doxorubicin in future studies.

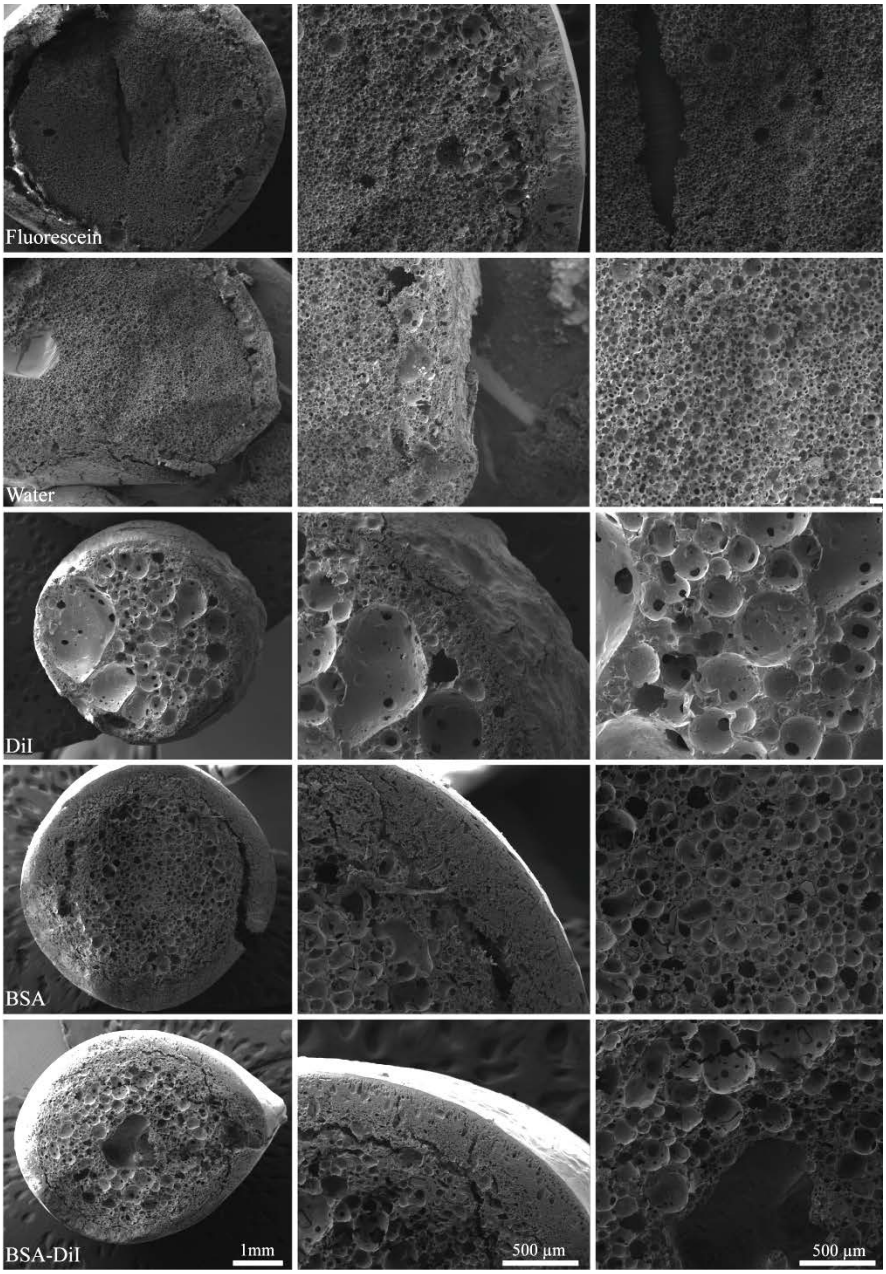

Figure 4: SEM images of implants highlighting the microstucture near the shell as well as the interior regions of the implants.

Chemotaxis studies and Receptor Levels–

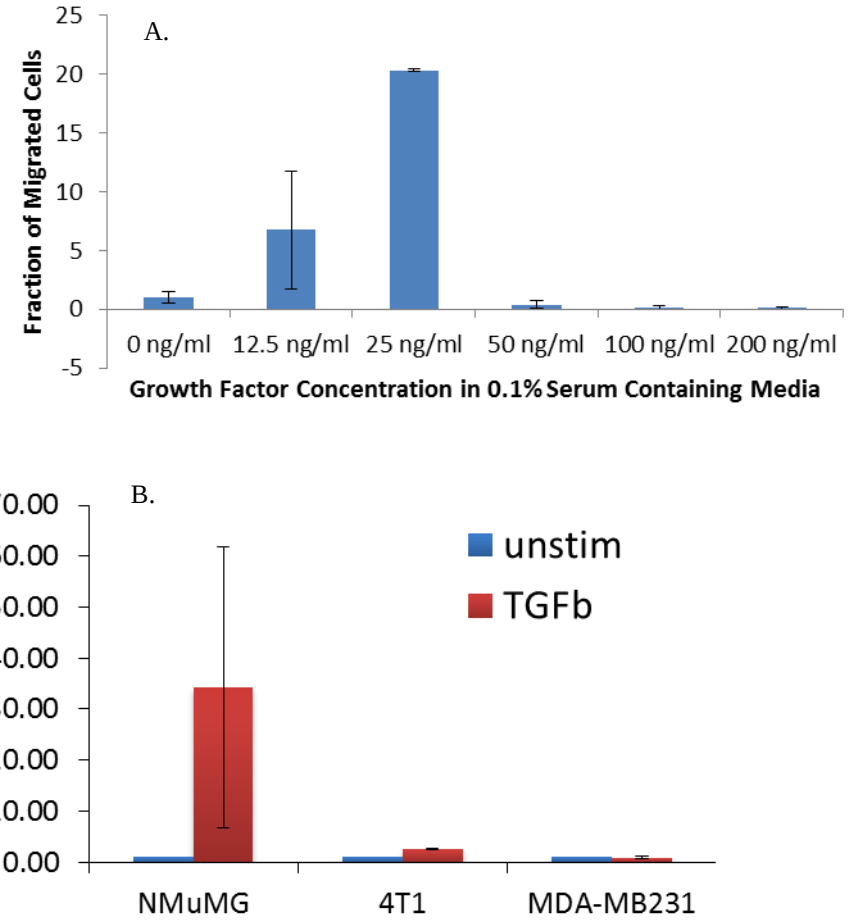

Figure 5: (**A**) Chemotaxis study of cells migrating in response to SDF-1. (**B**) Effect of TGF-β (TGFb) stimulation on CXCR4 receptor levels.

Migration assays of SDF-1 and EGF were performed, with EGF showing minimal migration (a maximum of 2 fold, relative to serum free wells, data not shown). These assays were then used to optimize loading conditions of gels, so that peak concentrations would be maintained within the implants (**Figure 5A**). All implants will be loaded with at least 25 ng/ml SDF-1 for future release studies. CXCR4 receptor levels were then evaluated in 3 cell lines. NMuMG a normal epithelial cells line, 4T1 a murine model for triple negative cancer, and MDA-MB-231 cells which are a human model for metastatic breast cancer. TGF-β was not selected for chemotaxis studies due to the significant increase it has on CXCR4 receptor levels on the normal/control cell line (**Figure 5B**)

3D Gradient Development and GFP positive cells–

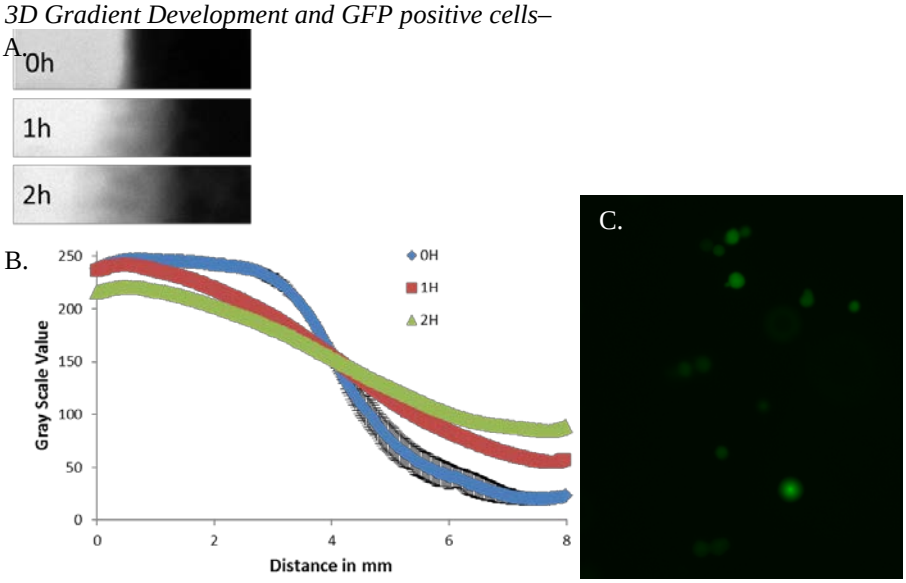

Figure 6: (**A**) Fluorescence image of dextran loaded gelatin diffusing over time. (**B**) Output of image processing on the fluorescence image, which can then be fit to a model of diffusion for predicative analysis of gradient development. (**C**) GFP-modified MDA-MB-231 cells.

Since cell migration to SDF-1 has been demonstrated to be a function of the chemokine gradient, a method for developing a growth factor gradient in 3D was developed. Gelatin density was controlled using sucrose, and then layered, with one layer containing a fluorescent 8kDa dextran (**Figure 6A**). The diffusion was then monitored over time using the Maestro imaging system, and subsequent images were evaluated using custom written image processing codes in MatLab (**Figure 6B**). The diffusion was then modeled so that a number of growth factor spatial distribution profiles could be developed and chemotaxis studies could be performed on the gradient gels. To prepare for the upcoming gradient studies, GFP positive MDA-MB-231 cells were made (**Figure 6C**)

Due to poor release and denaturation of SDF-1 from the polymer implants, the implant system has been changed to a polymer scaffold system using heparin to function as the means by which controlled release is achieved. PLGA is stacked through a process developed in the Lahann lab using electrohydrodynamic cojetting (**Figure 7**). These stacks can then be surface coated using chemical vapor deposition, and subsequently coated with extracellular matrix proteins such as laminin and fibronectin (**Figure 7**). Cells can either be cultured directly onto the scaffolds, or through the release of SDF-1, be directed from a surrounding matrix onto the scaffolds (**Figure 8-9**).

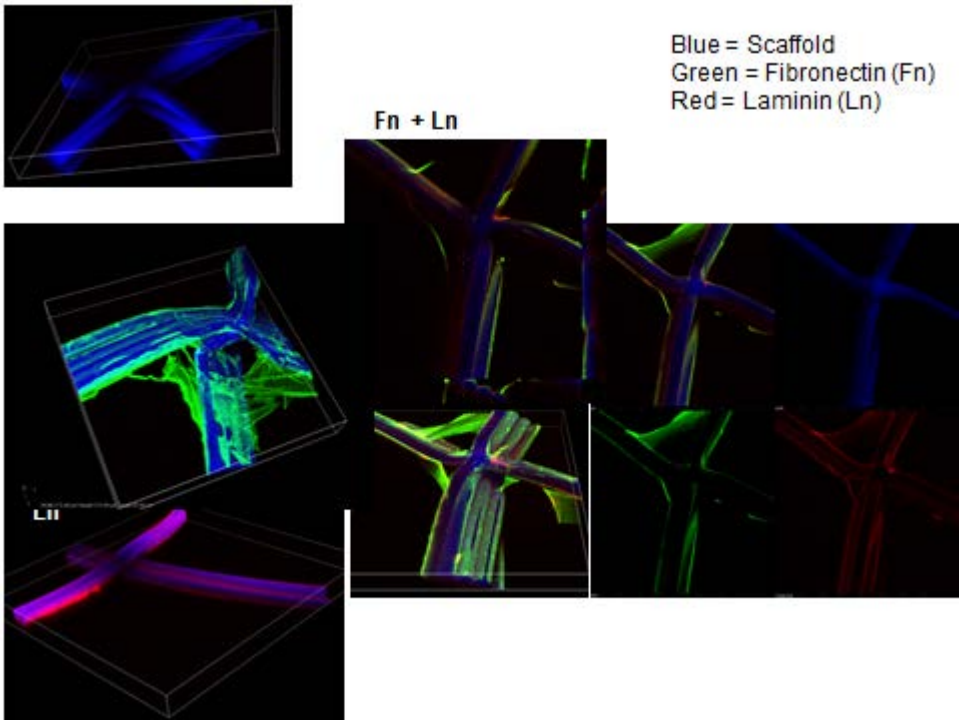

Figure 7: Blue stacked fibers are shown with antibody staining performed to demonstrate the potential for adhering extracellular matrix proteins onto the scaffold substrate. In this image laminin, fibronectin, and a combination of the two were used to coat the scaffolds.

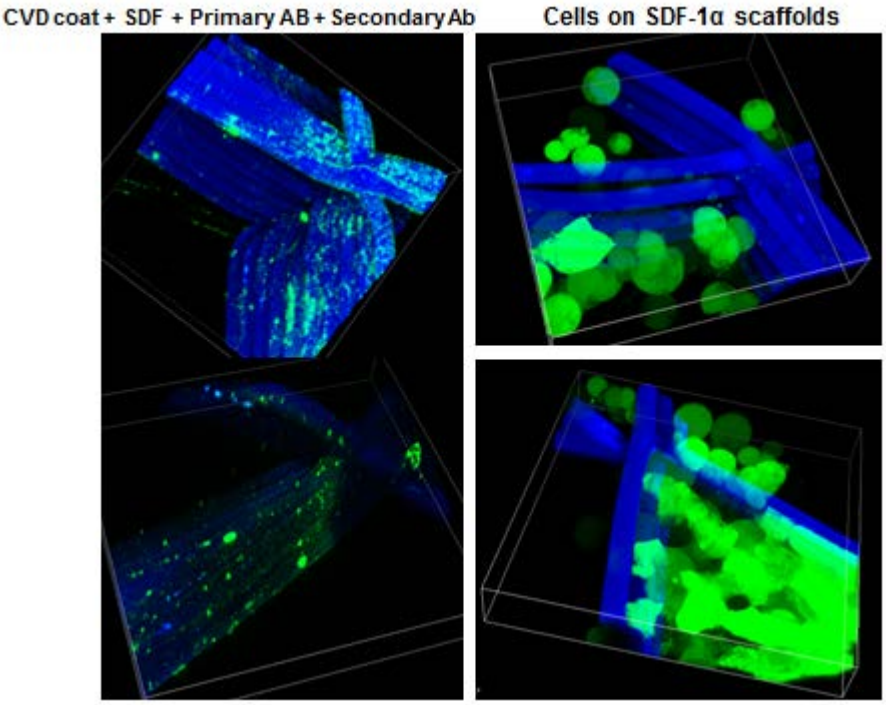

Figure 8: Chemical vapor deposition was performed to facilitate conjugation of SDF-1 onto the surface of the scaffold. The scaffold was then placed in a tissue culture dish, and allowed to function as a chemoattractant substrate, which resulted in the migration of cells onto the scaffold surface. In this instance no ECM proteins were adsorbed onto the scaffold.

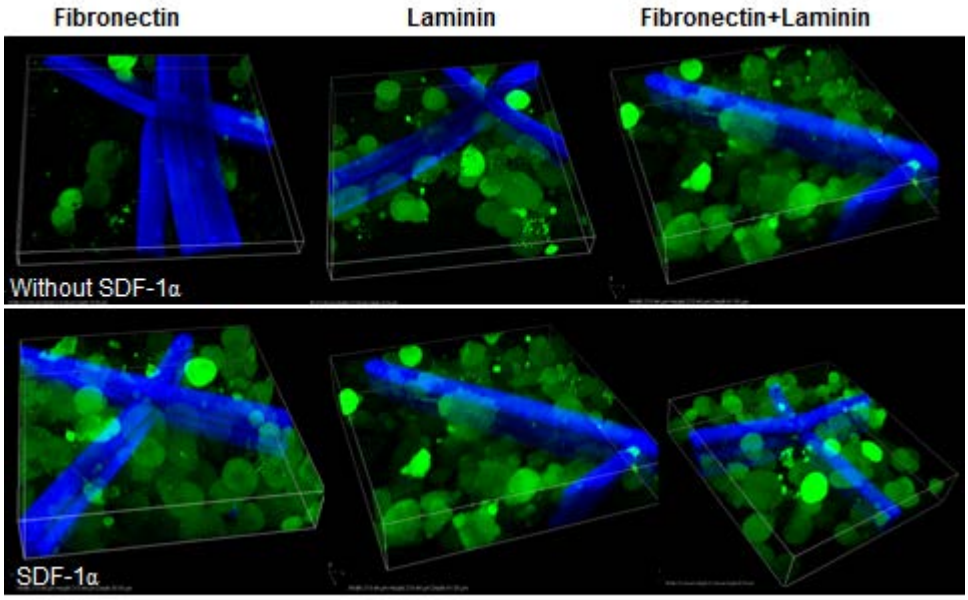

Figure 9: Confocal images of GFP expressing MDA-MB-231 cells grown on the scaffolding system after 4 days. Scaffolds with SDF-1 were first CVD coated in order to react SDF-1 onto the surface, then subsequently coated with the listed ECM proteins.

We observed that we can create metastatic niche through the use of SDF-1 and adsorption of ECM proteins. We can induce cell migration into the niche, and facilitate cell growth and expansion on the 3D substrate. We have begun incorporation of doxorubicin into the scaffolding fibers to evaluate the effectiveness of the system for killing the cancer cells. Additionally we have developed a means by which we can conjugate Heparin onto the surface of the polymer in order to facilitate controlled release of SDF-1 (**Figure 10**). SDF-1α will be predominantly released, with the varying ratios of the isoform SDF-1γ used to control the release profile due to the higher affinity of the γ isoform with heparin (**Figure 10**).

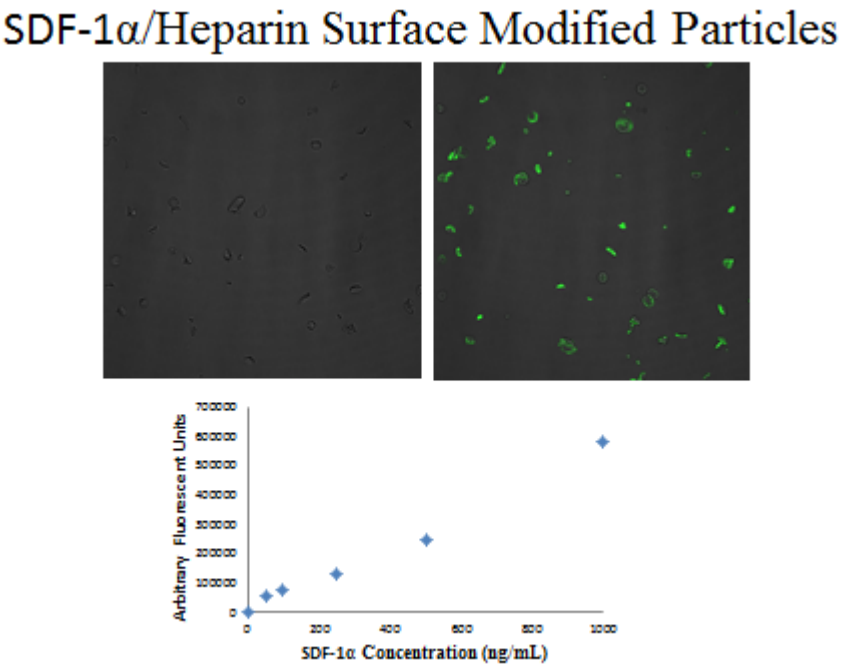

Figure 10: Antibody staining demonstrating SDF-1α association with PLGA particles surface modified with heparin. Left image is the control with no SDF-1α, with the right image having particles that contain SDF-1α. The graph demonstrates the effect of SDF-1α concentration on particle loading, demonstrating that there is a near linear response of particle loading as a function of SDF-1α concentration.

List of Completed Tasks:

- Application for IACUC protocol approved by CWRU, ACURO approval pending transition to UofM (month 6)
  -Protocol submission to U of M is pending approved finalized fellowship transfer to U of M
- Determine the optimal concentration and effective concentration range of chemoattractants (months 1-2)
- Evaluate release rate of doxorubicin , chemoattractant, and co-loaded implants (months 2-4, in progress)
- Evaluate effects of excipient loading on implant swelling and phase inversion in order to optimize implant release profile (months 3-4)
- Initiate culture of cell lines (months 3-4, ongoing)
- Developed a system to characterize the spatial distribution of drug within a 3D scaffold to determine the effect on chemotaxis (months 4-5, in progress)
- Transfect cells with GFP and confirm transfection (months 5)
- Developed novel system for establishing a metastatic niche using a PLGA fiber scaffolding system and extracellular matrix components
- Developed methods for effective release and conjugation of SDF-1α from the PLGA scaffold network

Section II – Reportable Outcomes

- The effect of excipients on drug release and phase inversion of phase inverting implants-*in preparation*
- The effect of environment on drug release and microstructure of phase sensitive in situ forming implants-*in preparation*
- Efficacy doxorubicin loaded *in situ* forming implants-*in preparation*

Section III – Problem Areas
Current problems and proposed corrective action

Current issues include challenges in evaluating SDF-1 gradients in a collagen gel. We are attempting to image SDF-1 conjugated with fluorescent dyes. The number of dye molecules conjugated to the growth factor is not significantly high to see with confocal microscopy. Therefore, we will be cyrosectioning the samples in order to use antibody staining to evaluate the distribution through the gels.

Anticipated problems and proposed corrective action

We anticipate that while we will get a tailorable release profile using heparin/SDF-1 interactions, without denaturation of the growth factor, loading may be insufficient to elicit a response *in vivo*. If the growth factor loading proves to be insufficient, then scaffolds will be seeded with SDF-1 expressing cells provided by Dr. Gary Luker in order to provide a constant source of the growth factor.

Section III – Conclusions and Upcoming Work

We have investigated methods for tailoring the release profile of *in situ* forming implants, and investigated the effect of protein loading on the phase inversion and drug release. Release of SDF-1α, was not conclusive, and indicated that the labile protein was denatured in these formulations. In order to limit this effect, a PLGA scaffolding system was developed, which will facilitate the delivery of active growth factor. This system can be used to evaluate the effect of ECM components as well as decouple the effect of modulus from matrix concentration through changes in the polymer fiber modulus. Additionally techniques for chemically conjugating the labile growth factor directly to the scaffold were developed. Techniques for releasing active growth factor, have been developed through the use of heparin. Ongoing studies are evaluating the release of SDF-1α from heparin coated scaffolds, and their chemotactic potential through a collagen gel. Additionally, these implants will be implanted in mice to determine the chemotactic potential *in vivo*.